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Abstract: The area postrema (AP), located in the caudal hindbrain, is one of the primary binding sites for the endocrine satiation hormone amylin. Amylin is co-secreted with insulin from pancreatic ß-cells and binds to heterodimeric receptors that consist of a calcitonin core receptor (CTR) paired with receptor-activity modifying protein (RAMP) 1 or 3. In this study, we aim to validate a CTR-floxed (CTRfl/fl) mouse model for the functional and site-specific depletion of amylin/CTR signaling in the AP and the nucleus tractus solitarius (NTS). CTRfl/fl mice were injected in the NTS with adeno-associated virus (AAV) containing a green fluorescent protein tag (GFP) and Cre recombinase to create a locally restricted knockout of CTR in the caudal hindbrain. KO mice showed a lack of c-Fos expression, a marker for neuronal activation, in AP, NTS and LPBN after amylin injection. The effect of amylin and salmon calcitonin (sCT), an amylin receptor agonist, on food intake was blunted in KO mice, confirming a functional reduction of amylin signaling in the hindbrain.

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Viral depletion of calcitonin receptors in the area postrema: A proof-of-concept study

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Highlights

- Depletion of calcitonin receptors in the area postrema is achievable by AAV injection
- Acute anorectic effects of amylin are completely abolished
- Linear correlation with receptor depletion allows detection of smaller effect sizes

ABSTRACT

The area postrema (AP), located in the caudal hindbrain, is one of the primary binding sites for the endocrine satiation hormone amylin. Amylin is co-secreted with insulin from pancreatic ß-cells and binds to heterodimeric receptors that consist of a calcitonin core receptor (CTR) paired with receptor-activity modifying protein (RAMP) 1 or 3. In this study, we aim to validate a CTR-floxed ($CTR^{fl/fl}$) mouse model for the functional and site-specific depletion of amylin/CTR signaling in the AP and the nucleus tractus solitarius (NTS). $CTR^{fl/fl}$ mice were injected in the NTS with adeno-associated virus (AAV) containing a green fluorescent protein tag (GFP) and Cre recombinase to create a locally restricted knockout of CTR in the caudal hindbrain. KO mice showed a lack of c-Fos expression, a marker for neuronal activation, in AP, NTS and LPBN after amylin injection. The effect of amylin and salmon calcitonin (sCT), an amylin receptor agonist, on food intake was blunted in KO mice, confirming a functional reduction of amylin signaling in the hindbrain.

INTRODUCTION

Adeno-associated virus (AAV) vectors are a viable tool to study site-specific alterations in central control of metabolism (Mancini and Horvath, 2018). In this study, we used an AAV that expresses Cre recombinase in its target cells under the human synapsin 1 (hSyn1) promoter to remove the loxP-flanked exons 6 and 7 of the calcitonin receptor (CTR) gene in $CTR^{fl/fl}$ mice (Keller et al., 2014). CTR is the core component of the amylin receptor, which is a heterodimer of CTR and receptor activity-modifying proteins (RAMP) 1-3 (Christopoulos et al., 1999, McLatchie et al., 1998). The genetic depletion of CTR in the target areas is expected to affect all subtypes of the amylin receptor and diminish any effect of the ligand conveyed through this receptor. Amylin is a peptide hormone, co-released from the pancreas with insulin, and has direct central effects on food intake and energy expenditure by acting on noradrenergic neurons in the area postrema (AP) and pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC) (Lutz et al., 2018, Potes et al., 2010b, Pan et al., 2018). The effect of amylin on the AP to acutely reduce food intake has been extensively studied (Barth et al., 2004, Liberini et al., 2016, Lutz et al., 1998) and amylin-induced c-Fos has been demonstrated to be a marker of amylin's neuronal activation in the AP and LPBN (Becskei et al., 2007)

Hence, amylin's activation of c-Fos serves as a verification for successful CTR depletion after AAV injection in $CTR^{fl/fl}$ mice. Although the use of viral vectors in circumventricular organs comes with technical difficulties, a recent study successfully applied shRNA-delivery into the AP by injecting the virus into the adjacent nucleus of the solitary tract (NTS) (Tsai et al., 2019). In this study, we use the same approach to allow AAV-Cre expression in AP neurons for CTR depletion and measure the acute effects of amylin and its agonist salmon calcitonin (sCT) on food intake and c-Fos expression to quantify the loss of function.

MATERIAL AND METHODS

CTR$^{fl/fl}$ mice and housing

CTR$^{fl/fl}$ mice (*Calcr<tm1(fl)>;* MGI:5751436; frozen sperm was kindly provided by Dr. Jean-Pierre David and Dr Thorsten Schinke, University Medical Center Hamburg) (Keller et al., 2014) were bred for the experiment and distributed into age-matched treatment groups. The animals were kept in a controlled environment (21±2°C, 12/12 inversed light cycle) and on a standard chow diet (3430, Provimi Kliba, Kaiseraugst, Switzerland) for the entire experimental period. Three weeks of habituation/recovery were provided before and after surgery. Male mice were single housed after surgery because of fighting while female mice were single housed one week before the start of food intake measurements. All procedures were approved by the Veterinary Office of the Canton Zurich, Switzerland.

Ai14 reporter mice *[B6.Cg-Gt(ROSA)26Sor<tm14(CAG-tdTomato)Hze>/J(#007914)]* were crossed with CTR$^{fl/fl}$ mice for pilot studies to confirm stereotaxic coordinates and AAV-Cre induction. Expression of Cre recombinase leads to the removal of a LoxP-flanked STOP cassette that prevents CAG-promoter-driven transcription of red fluorescence protein (tdTomato) in this reporter line (Madisen et al., 2010).

Surgical procedures for virus delivery

Nine- to fifteen-week-old male and female mice were anesthetized by intraperitoneal injection of ketamine (Ketanarkon-100, Streuli Pharma; 75mg/kg) and medetomidine (Medetor, Virbac; 0.75mg/kg). The head was fixed with intraaural pins to a stereotaxic platform (KOPF instruments, Tujunga, CA). Burr holes were drilled at the target coordinates and a 35 G syringe (World precision instruments, Sarasota, FL) was inserted for a bilateral injection of 0.8µl AAV-Cre or AAV-GFP each. The target coordinates for the NTS were -7.5mm posterior for males, 7.45mm posterior for females and 0.25mm lateral from bregma, with a depth of 4.5mm, measured from the skull surface (modified from (Tsai et al., 2019)).

Anaesthesia was antagonized by intraperitoneal injection of atipamezol (Revertor, Virbac; 3.75mg/kg). Mice received antibiotic and analgesic treatment on surgery day and the following three days with subcutaneous injections of enrofloxacin (Baytril, Bayer; 7.5mg/kg) and meloxicam (Metacam, Boehringer Ingelheim; 1mg/kg).

Viral vectors for stereotaxic injection

Viral vectors were obtained from the repository of the core facility at the University of Zurich and ETH: AAV-Cre (v146-8; ssAAV-8/2-hSyn1-chI-EGFP_2A_iCre-WPRE-SV40p(A), physical titer 1.1*10E12 vg/ml) and AAV-GFP (v308-8; ssAAV-8/2-hSyn1-chI[4x(shNS)]-EGFP-WPRE-SV40p(A), physical titer 4.1*10E12 vg/ml).

Food intake measurements

Mice were single-housed and adapted to food hoppers in BioDAQ cages (Research Diets, New Brunswick, NJ) over a one-week period. Baseline food intake was then measured for three days with ad libitum access to chow. Food intake in response to amylin was assessed in a crossover design with a three-day washout period, with intraperitoneal injections of amylin (H-9475, Bachem, Bubendorf; 50, 500µg/kg) or saline (NaCl 0.9%) at lights out after a 12h fast. Four female KO mice were excluded from the following measurements after displaying clinical symptoms of discomfort or maladaptation to single housing. The food intake after the CTR agonist salmon calcitonin (4033011.0001, Bachem; 10µg/kg i.p.) was measured at the end of the study in the remaining animals to exclude interference of this longer acting CTR agonist with other measurements. Individual meals were defined with an inter-meal interval of ≥600s and a meal size of ≥0.02g (Duffy et al., 2018).

Perfusion and brain processing

Mice were fasted for 12h during the light phase and injected with amylin (50µg/kg i.p.) at dark onset 90 minutes prior to perfusion. After terminal anaesthesia with pentobarbital (Kantonsapotheke Zurich, Switzerland; 200mg/kg i.p.), mice were perfused intracardially

with cold 0.1M phosphate buffer (PB) for 1.5min followed by 4% paraformaldehyde (PFA) in PB for 2.5min. The brains were postfixed for 24h in 4% PFA and cryoprotected for 24h in 30% sucrose before being frozen in hexane on dry ice for storage at -80°C. Sections of 20µm were cut on a cryostat (CM3050S, Leica Biosystems, Germany), mounted on Superfrost Plus slides (Thermo Fisher Scientific, Reinach, Switzerland) and cryoprotected in 50% 0.02M potassium phosphate buffered saline (KPBS), 30% ethylene glycol, 20% glycerol for storage at -20°C.

Immunohistochemistry

c-Fos IHC of AP, NTS and LPBN (Fig. 4): Brain sections were rinsed five times in 0.02M KPBS before an overnight block in 5% normal goat serum (NGS) + 0.4% Triton in KPBS at 4°C. The sections were then placed in primary antibody solution (1:500 rabbit anti-c-Fos, *Cell Signaling #2250* in KPBS + 0.4% triton and 2% NGS) for 48h at 4°C. After five rinses in KPBS, sections were placed in secondary antibody solution (1:100 goat anti-rabbit Cy3, *Jackson #111-165-144* in KPBS + 0.4% triton and 2% NGS) for 2h at room temperature (RT). Sections were counterstained with DAPI (0.25µg/ml, 5min at RT) and cover-slipped with vectashield (Vector Laboratories).

CTR IHC of AP and NTS (Fig. 1): Brain sections were rinsed five times in 0.01M PBS + 0.1% triton, blocked for 2h at RT in 3% NGS + 0.3% triton in PBS and placed in primary antibody solution (1:500 rabbit anti-CTR, *Abcam #ab11042* + 0.3% triton) for 48h at 4°C. After two rinses in PBS and one rinse in PBS + 0.1% triton, sections were placed in secondary antibody solution (1:250 goat anti rabbit 647, *Invitrogen #A-21245* + 0.3% triton) for 2h at RT and counterstained with DAPI (Duffy et al., 2018).

CTR IHC for quantification (Fig. 5): The sections for CTR quantification were stained with a modified protocol that includes antigen retrieval (0.01M citric acid pH 6 + 0.05% Tween 20 in steamer for 20 min) and amplification of secondary antibodies (1:1000 biotinylated goat

anti rabbit, *Vector #BA-1000* + 0.3% triton for 2h at RT, three rinses with PBS + 0.1% triton, 1:1000 streptavidin-647, *Invitrogen #S21374* + 0.3% triton for 2h at RT) to increase sensitivity (Coester et al., 2020).

Imaging and quantification

Immunostained sections were visualized with an Axio Imager 2 microscope (Zeiss Germany) and three sections of AP/NTS (bregma -7.31 to -7.55) and LPBN (bregma -5.19 to -5.33) per animal were recorded for cell counting. Grayscale pictures of c-Fos staining (Fig. 4) were converted to binary with a pre-defined threshold in ImageJ and positive cells were counted with the "analyze particles" utility (size = 10-infinity; circularity 0.10-1.00)(Schindelin et al., 2012). Pictures of CTR staining (Fig. 5) were quantified by manual annotation to exclude fibers and only count cell bodies.

Statistics

All data was analyzed with Prism 8 (GraphPad, La Jolla, CA). Body weight data was separated by sex and analyzed with a mixed effects model for repeated measures and matched values (Fig. 2A-B). Meal patterns were analyzed using repeated measure analysis of variance (ANOVA) and multiple comparisons with Sidak correction (Fig. 2C-E). Food intake data was analyzed with two-way ANOVA and multiple comparisons with Bonferroni's correction (Fig. 3). Cell quantifications for c-Fos and CTR were analyzed with two-way ANOVA and multiple comparisons with Sidak's correction (Fig. 4B, 4D, 5B). Correlations of CTR quantification and food intake or meal patterns were analyzed by simple linear regression (Fig. 5 C-F). Grouped data are expressed as mean ± SEM with individual data points indicated, and any statistically significant differences between groups are indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$), non-significant trends are annotated with the exact p value. Results of simple linear regression analysis are individually annotated.

RESULTS

Bilateral NTS injections allow Cre-mediated recombination in the AP

9-week-old $CTR^{fl/fl}$ x $Ai14^{Tg/+}$ mice were injected with AAV-GFP (control; Fig. 1A) or AAV-Cre (KO; Fig. 1B), respectively. Both groups show robust GFP expression at the injection site in a pilot experiment two weeks after surgery (Fig. 1C and D, top). AAV-Cre-injected mice additionally show tdTomato fluorescence in the AP following Cre-mediated recombination, which confirms expression of Cre recombinase after virus injections. CTR immunohistochemistry revealed no co-localization of CTR fibers with tdTomato/GFP-positive cells in KO mice and abundant co-localization of CTR fibers with GFP in control mice (Fig. 1C). The adjacent NTS also shows robust GFP expression but little detectable CTR immunofluorescence.

Mice retain a stable body weight after virus injection

9- to 15-week-old male and female $CTR^{fl/fl}$ mice underwent surgery for virus injection and were allowed to recover for three weeks. Male mice had to be single housed after surgery because of aggression, female mice stayed in group housing until the food intake measurements 6 weeks after surgery (Fig. 1D). There was no significant difference in body weight or body weight gain between treatment groups (Fig. 2A-B). Meal patterns at week 6 after surgery were analyzed in a three-day average by number of meals, average meal size and average meal duration, but no significant effects were found when comparing KO to control groups under baseline conditions (Fig. 2C-E). When CTR expression and meal duration are correlated in the KO group only, meal duration is significantly increased when CTR are depleted (Fig 5C).

KO mice are insensitive to amylin and salmon calcitonin injections

When KO mice are compared to control mice after injection of amylin (50 or 500µg/kg) or sCT (10µg/kg), there is a significant effect for genotype in 4-hour cumulative food intake

(Fig. 3C) and a non-significant trend for treatment effect at 4 and 12 hours after injection (Fig. 3C-D). When cumulative food intake is calculated as percentage of saline, a significant effect between genotypes is observed at 2, 4 and 12h after injection (Fig. 3F-H). Post-hoc analysis shows that the genotype effect is mainly driven by a higher food intake of KO mice after sCT injection (Fig. 3G-H).

c-Fos expression in response to amylin is abolished in the AP, NTS and LPBN of KO mice

Amylin injections (50µg/kg) after overnight fasting elicit a strong c-Fos response in the AP and NTS of control but not KO mice (Fig. 4A-B). Both groups show robust GFP expression 8 weeks after virus injection, but only control mice show c-Fos expression in GFP-labelled cells after amylin injections (Fig. 4A). GFP-labelled neuronal fibers are also present in the LPBN, a downstream nucleus of amylin signaling in the hindbrain (Fig. 4C). LPBN c-Fos expression after amylin injection is significantly increased in control mice compared to saline (Fig. 4D).

Depletion of CTR correlates with increased food intake after amylin injection and increases meal duration

AP and NTS were stained for CTR to quantify expression at the end of the study (Fig. 5A). Quantification of CTR at the end of the study showed a successful depletion in 56% of the animals for the AAV-Cre group (Fig. 5A-B). Histograms of CTR+ cells in the AP showed two clusters that were separated into “Hit” and “Non-Hit” groups to exclude data of KO mice with abundant CTR expression from grouped analyses (Fig. 5B). Quantification of CTR in the NTS revealed only sparse expression in all groups. Correlation of CTR+ cells with meal pattern data showed a significant association of CTR depletion and meal duration, but not meal number (Fig. 5C). KO mice injected with amylin showed a significant association of

CTR depletion and food intake at 2h post-injection (50µg/kg; Fig. 5E) and this correlation was highly significant at 2h and 4h after a higher dose of amylin (500µg/kg; Fig. 5F).

DISCUSSION

In this study, we aimed to validate the site-specific depletion of CTR using AAV-Cre in a $CTR^{fl/fl}$ mouse model to confirm a functional reduction of amylin signaling after the loss of CTR in AP neurons. This pathway was chosen because all necessary amylin receptor components are present in single AP neurons (Liberini et al., 2016) and downstream signaling to the LPBN has been linked to feeding-related changes after amylin injection (Riediger et al., 2004). The pharmacological depletion of amylin signaling or lesion of the AP increase food intake and abolish amylin-induced food intake reduction (Lutz et al., 2001, Mollet et al., 2004).

In a first experiment, an Ai14 Cre reporter mouse was crossed with $CTR^{fl/fl}$ and injected with AAV-Cre virus into the NTS to assess Cre expression in the AP. The AAV-Cre virus codes for both GFP and Cre recombinase, separated by self-cleaving peptide 2A, while AAV-GFP only codes for GFP. The relative intensity of GFP and tdTomato fluorescence is variable, since the former depends on the initial transfection and the latter depends on CAG-promoter-driven expression after the LoxP-flanked STOP-cassette is removed (i.e. successful Cre-mediated recombination) (Madisen et al., 2010).

Earlier experiments with direct injection into the AP have been mostly unsuccessful, probably due to insufficient retainment of virus suspension in the circumventricular organ, which is heavily vascularized. A recently published study by (Tsai et al., 2019) took a different approach by injecting the virus into the adjacent nucleus of the solitary tract (NTS). The retainment in the NTS and diffusion into the AP seem to be more successful for the transfection of AP neurons. The Ai14 reporter line confirmed Cre recombination in AP

neurons even in cells where GFP expression was faint, but diffusion to the AP was weak in the central part. The reason for this was determined to be a rostral offset of the injection site in the pilot experiment, therefore bregma coordinates were adjusted by 220 µm caudally for the main study.

Body weight of the mice was stable throughout the study and no difference between groups was found at the end of the study when all mice had fully regained their initial body weight. Surprisingly, there was no effect of CTR knockout in AP/NTS on meal patterns unlike full body knockout of RAMP1/3, that results in fewer, but larger and longer meals (Coester et al., 2019). Since these effects observed in RAMP 1/3 KO mice appear to be stronger in male mice, they could be masked in this predominantly female study. Interestingly, when CTR depletion was correlated with meal patterns in the AAV-Cre group, we observed that the CTR depleted animals had significantly longer meals, which is in line with previous data and confirms the function of amylin as a satiation factor (Lutz et al., 1995).

Cumulative food intake after overnight fasting and refeeding with injections of amylin (50 and 500µg/kg) seemed to be initially reduced in KO mice in the first hour, but these trends are difficult to interpret due to the high variance of 1h food intake in mice. At 2h and 4h, where we usually observe an effect of amylin on food intake (Coester et al., 2019), KO mice showed no response to amylin or sCT. Conversely, amylin and sCT significantly decreased food intake in control mice when expressed as percent of saline. These observations can be recapitulated when correlating CTR depletion with food intake after amylin injection in AAV-Cre mice. Depleting CTR significantly correlated with increased food intake after 2h and 4h following the higher dose of amylin and 2h after the lower dose of amylin, further confirming the role of amylin AP/NTS signaling as a major hub for meal termination (Lutz, 2012). As for sCT, this regression analysis is inconclusive due to the exclusion of four female KO mice prior to the measurement. All four excluded mice were CTR-depleted (i.e. in the

AAV-Cre "Hit" group) and showed signs of maladaptation to single housing. We could hypothesize that due to the previously proposed anxiolytic properties of amylin (Roth et al., 2009), these KO mice were more susceptible to stress and had increased anxiety.

Since amylin is known to elicit a strong c-Fos response in AP/NTS neurons, amylin injections 90 minutes prior to sacrifice are routinely used to quantify amylin signaling histologically (Potes and Lutz, 2010). GFP expression in the AP/NTS was robust in both groups and there was virtually no overlap of GFP and c-Fos in the KO group. Quantification of c-Fos in the AP and NTS confirmed that KO mice do not respond to amylin injections with a measurable increase of c-Fos-positive cells. The LPBN is innervated by AP neurons (Riediger et al., 2004, Potes et al., 2010a) and GFP-positive fibers encapsulating second-order neurons were found in the dorsal and external part of the LPBN. Control mice showed a significant increase in c-Fos-positive cells after amylin injection while KO mice only show a marginal increase, which confirms a functional depletion of amylin signaling in the hindbrain axis.

The quantification of c-Fos does not always completely correlate to the food intake data (Duffy et al., 2018, Watts et al., 2006), and since c-Fos does not directly depict amylin action in the AP, it is possible that a marginal activation is overlooked with this method (Potes and Lutz, 2010). However, the analysis of food intake after amylin injection was more sensitive (i.e. may be observed at lower doses) when CTR depletion in AAV-Cre mice was compared to food intake, and it showed a significant effect of the lower amylin dose (50µg/kg) that was also used for c-Fos. It seems that this internal control unintendedly worked better than the actual AAV-control group and therefore the non-significant trends in group analyses are just lacking statistical power due to the smaller effect size. The reason for this is possibly the wider distribution of CTR+ cell abundance in AAV-control mice, which had significantly a different standard deviation compared to the AAV-Cre groups.

Overall, these data confirm that bilateral injection of AAV in the NTS is a valuable technique to avoid previous issues with viral injection into circumventricular organs like the AP. The successful viral depletion of CTR shows that $CTR^{fl/fl}$ mice can be used for site- and cell type-specific knockouts of CTR to study central amylin signaling pathways. The problem remains that this approach does not allow a distinction between AP and NTS that would allow a confirmation of NTS specific effects of CTR, as it was proposed in a recent study (Cheng et al., 2020). This is especially intriguing since we see only sparse CTR expression in the NTS of $CTR^{fl/fl}$ mice, which suggests that NTS neurons are mainly indirectly activated by amylin in this mouse model. Moreover, the use of Cre driver lines might obscure that CTR expression is subject to post-translational regulation. We have previously shown that the knockout of RAMP 1/3 and RAMP 3 alone is sufficient to reduce CTR expression in the AP (Coester et al., 2019).

The meal pattern data highlights that not all phenotypic effects of RAMP1/3 KO can be directly reproduced with site-specific CTR KO. Possibly, the unconditional RAMP1/3 KO affects more than just the hindbrain signaling of amylin (Hay et al., 2005), and meal patterns could be influenced by other brain areas.

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